POLYDEOXYTHYMIDYLATE AS TEMPLATE FOR COMPLEMENTARY ENZYMATIC SYNTHESIS

F.N. HAYES, U. HOLLSTEIN, V.E. MITCHELL and R.L. RATLIFF
Biomedical Research Group, Los Alamos Scientific Laboratory, University of California, Los Alamos, New Mexico 87544, USA

and

Department of Chemistry, University of New Mexico, Albuquerque, New Mexico 87106, USA

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1. Introduction

Calf thymus DNA polymerase [1] catalyzes the polymerization of dATP in the presence of polydeoxythymidylate, dT_n. The polydeoxyadenylate segment is attached by phosphodiester linkage to the 3′-end either of the dT_n or of an added oligodeoxyadenylate initiator [2]. In contrast, an absolute requirement for exogenous initiation is characteristic of *Micrococcus luteus* DNA polymerase [3]. For both enzymes the reaction does not proceed beyond 1-fold synthesis. However, various extents of reaction (1-fold and less) have been reported [2–4]. One explanation for early conclusion of the reaction is formation of a stable triple-stranded structure involving two strands of dT_n and one of dA_n.

The triple-stranded complex $dA_n \cdot 2dT_n$ has been observed on physical mixing of the single strands in the proportion 1 dA_n to 2 dT_n at high sodium ion concentration where n was in the range 3,000–6,000 nucleotide units [5]. The product was characterized by its two-step thermal hyperchromicity profile at 260 nm, for which the T_m values in 0.2 M Na^+ are 43° and 74.5°, by equilibrium gradient centrifugation in cesium sulfate, and by a thermal transition that occurs at 284 nm for triple- to double-stranded and not for double- to single-stranded. The molecular structure of the polymerase-catalyzed, tripled-stranded product may have two quite different forms. One structure exists because half of the dT_n strands have completed 1-fold synthesis and are associated with the remaining half of the dT_n in a triple-stranded structure. Alternatively, 0.5-fold synthesis has occurred on all of the dT_n strands, and the single-stranded portion of the dT_n has looped back upon the double-stranded structure to give the triple-stranded product.

This work is concerned with determination of the structures and finding how the experimental conditions influence their formation. All syntheses with a dT_n template were run without initiator to aid identification in that the newly-synthesized segment cannot depart from the template.

2. Materials and methods

Terminal deoxynucleotidyltransferase [1] was used [6] to polymerize dTTP onto tritium labeled dT_4 [7], resulting in dT_n samples with average n values of 110, 170, and 460 as determined by radioisotopic dilution [6] and by agarose column chromatography [8]. Similarly, dATP and dA_6 gave dA_{240}.

Calf thymus DNA polymerase [1], 756 units/mg (1 unit = 1 nmole of dTTP polymerized per mg of protein per hr at 37° using dT_6A_{100}, dT_6, and dTTP-2-^{14}C), was titrated in a series of reactions at 15° in 40 mM potassium phosphate buffer (pH 7.0), conditions under which the polymerase does not recycle after completing one round of 1-fold synthesis. The

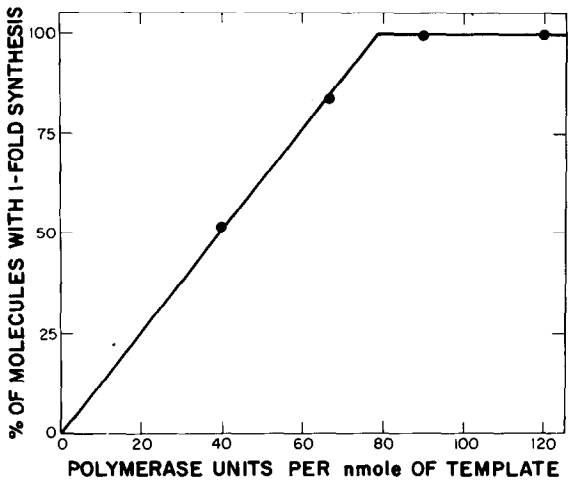


Fig. 1. Titration of calf thymus DNA polymerase with dA_{240} at 15°. Each reaction contained 4.5 μM dA_{240}, 4.8 μM dT_{10}, 2.5 mM dTTP-2-^{14}C (1,600 dpm/nmole), 8 mM $MgCl_2$, 1 mM 2-mercaptoethanol, and 45 mM potassium phosphate buffer (pH 7.0) plus calf thymus DNA polymerase as indicated in the legend of the abscissa, all in 0.10 ml volume.

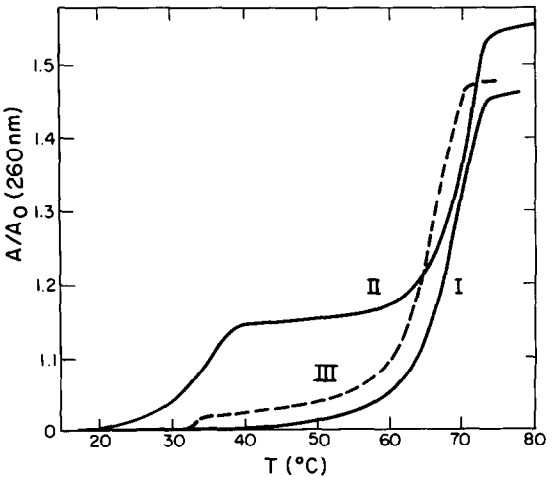
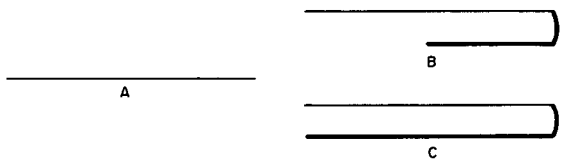


Fig. 2. Melting profiles of polydeoxythymidylate–polydeoxyadenylate complexes. The temperatures on the abscissa are corrected and were measured in the solution as it was simultaneously heated and analyzed spectrophotometrically at 260 nm [13]. The solutions contained 0.1 M NaCl and 0.05 M Na_2HPO_4 (pH 7.0, adjusted with H_3PO_4) in a total volume of 1.0 ml. Solution I also contained 0.59 μM $d(T_{110}–A_{110})$. The polymer in solution II was from the 15° reaction; solution III was from the prematurely stopped reaction.

reactions employed dA_{240}, dT_{10}, and dTTP-2-^{14}C and were allowed to proceed until no further synthesis was measurable using the filter paper disc method [9]. By varying the amount of polymerase for each reaction the equivalence point was determined (fig. 1) to be 78.5 units per nmole of template. If one polymerase molecule binds to one template molecule, a molecular weight of 103,800 daltons can be calculated – in agreement with the value of 110,000 for the same enzyme activity determined by Sephadex G-200 column chromatography [10].

A series of reactions using dT_{110}, dT_{170}, or dT_{460} were run for a minimum of 24 hr at 37° in 2.8 ml total volume containing 5 μM template, a 5-fold excess of dATP-8-^{14}C over that required for 1-fold synthesis, 8 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 40 mM potassium phosphate buffer (pH 7.0), and calf thymus DNA polymerase in equivalence ratios to template (R) of 4, 2, 1, 0.5, 0.3 and 0.2. The reactions were monitored for completion of synthesis by filter paper disc method [9]. Protein was removed by phenol extraction [11], and agarose column chromatography in 5 mM NaCl [9] separated all polynucleotide material from low molecular weight substances. Each isolated polymer was analyzed for phosphorus content [12], UV absorption, radioactivity, and thermal hyperchromicity [13], fig. 2. Two further reactions were run as above except that R values were 1 and 0.33, the potassium phosphate buffer (pH 7.0) was 0.15 mM, and 3 days were needed for completion.

Having determined that all these reactions had gone completely 1-fold, two identical reactions were run at 37° and R = 2, stopping one when it had reached an estimated 0.5-fold synthesis and letting the other proceed to completion. Finally, one reaction at 15° was run at R = 0.3. By filter paper disc analysis it appeared that termination of synthesis occurred at about 0.35-fold synthesis.



COMPLETION	EQUIVALENCE	RECYCLING	PRODUCTS
–	+	+ OR –	B
–	–	+ OR –	A + B
+	+	+ OR –	C
+	–	+	C
+	–	–	A + C

Fig. 3. Relationship of conditions to products in uninitiated calf thymus DNA polymerase reactions utilizing dT_n (A) as template with dATP. The dA units are represented by the heavy lined portions of the 0.5-fold synthesis product B and the 1-fold C. Lack of completion refers to purposeful or accidental premature termination of reaction either abruptly or by depletion of an essential ingredient. Equivalence and recycling refer to the polymerase in quantity and incubation temperature used.

3. Results and discussion

All reactions at 37° that were allowed to proceed to completion gave products with specific radioactivity indicating a dA/dT ratio of 1.0 ± 0.1, with agarose column chromatography showing molecular weight approximately double that of the template used, and with a single-step melting profile (curve I, fig. 2). By these criteria the products have the structure dT_n–dA_n. The product from the reaction using insufficient polymerase at 15°, a temperature that inhibits recycling, gave a dA/dT ratio of 0.38 and the melting profile of curve II, fig. 2. Such results suggest that the product is the mixture dT_n–dA_n + dT_n. This same profile was obtained from an equimolar physical mixture of dT_n with the material that gave curve I. The product from the reaction that was prematurely stopped showed (curve III, fig. 2) only a trace of a low temperature transition followed by a sharp melting at about 3° below that of curve I. The analytical value for dA/dT was 0.47. The structure of this product can be written dT_n–$dA_{0.47n}$; the lowered T_m of the large hyperchromicity transition is due to the shortened continuous length of dA · dT hydrogen bonding.

The conclusions of this study are given in fig. 3. There are four types of products or product mixtures: B, in which the reaction was prematurely terminated, shown here arbitrarily as approximately 0.5-fold synthesis; C, in which 1-fold synthesis was completed on all template strands; A + B, in which both premature termination and insufficient polymerase left some unreacted A along with B; and A + C, in which insufficient polymerase under non-recycling conditions gave 1-fold synthesis on only part of the original A. All of these cases have been encountered in this work, and in each case the experimental conditions seem to account for the nature of the product according to the logic of fig. 3.

Cases B and A + C both have the potential for existing in a triple-stranded structure. Looping back of the single-stranded portion of B would form a structure in the polarity arrangement with T_1 antiparallel to both A and T_2, where T_1 and A represent the dT_n and dA_n segments held together by Watson-Crick [14] hydrogen bonding, and T_2 is another segment of dT_n functioning as the third strand. The preferred structure of Miles [15] for $rA_n \cdot 2rU_n$ has U_1 and U_2 both antiparallel to A. Construction with CPK atomic models of each of the T_1 and T_2 both antiparallel to A and the T_1 antiparallel to both A and T_2 structures showed that the former or Miles structure was superior in both steric freedom and separation of phosphate groups. Only the A + C product can readily assume the Miles structure; it gave the two-step melting profile (curve II, fig. 2). The intramolecular triple-stranded structure from B is not of the Miles polarity; the melting profile is (curve III, fig. 2) almost entirely the single-step, double- to single-stranded transition.

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